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REVIEW

Exhibit A

The Evolution of the Type I Interferons[1]

R. MICHAEL ROBERTS,[2] LIMIN LIU,[3] QUINGTAO GUO,[2] DOUGLAS LEAMAN,[4] and JAMES BIXBY[2]

ABSTRACT

There are five recognized subtypes within the type I interferons (IFN), IFN-α, IFN-β, IFN-δ, IFN-ω, and IFN-τ, although others may remain to be described, and the IFN-ω may have to be subdivided further because of their evident structural complexity. Together, they constitute an ancient family of intronless genes, possibly present in all vertebrates. THe *IFNA/IFNB* genes originated by duplication of a progenitor after the divergence of birds, most probably about 250 million years ago (MYA). The avian gene itself proceeded to duplicate to form a series of independent subtypes. The *IFND*, to date described only in the pig, arose from the *IFNA* lineage before the emergence of mammals about 180 MYA and might, therefore, be generally distributed in present day species. The *IFNB*, which occurs as a single gene in primates and rodents, have been duplicated in some other orders. Recent events have produced 10 or more genes in bovid species. The *IFNA*, which are clustered with the *IFNW* in humans and cattle, exist as multiple genes in all mammals so far examined as a result of a series of duplication events, some of which occurred recently and, therefore, independently in separate mammalian lineages. The *IFNW* diverged from the *IFNA* approximately 130 MYA, just prior to the emergence of mammals, and have continued to duplicate since then. The *IFNT*, which play a role in reproduction of ruminants, arose from an *IFNW* within the Artiodactyla suborder about 36 MYA and are found only in the suborder Ruminantia. These genes have also continued to duplicate to form an extensive family. Consequently, their involvement in early pregnancy is a feature of ruminants and not of other mammalian species.

INTRODUCTION

THE INTERFERONS (IFN), AS ORIGINALLY DEFINED by their ability to induce an antiviral state in their target cells, can be divided into two groups, type I and type II, that bear little discernible evolutionary relationship to each other.[1,2] The type II, or IFN-γ, have been described only in mammals. They are represented in each species thus far examined by single genes, and these genes possess introns. The type I IFN, the subject of this review, are encoded by multiple intronless, apparently closely linked genes. In mammals, they can be subdivided further into at least five subtypes,[3,4] the IFN-α, IFN-β, IFN-ω, IFN-τ, and IFN-δ, on the basis of differences in primary structure, antigenic cross-reactivity, and, as discussed later, likely time of evolutionary divergence.

The IFN were first discovered in chick embryonic tissue over 40 years ago,[5,6] although the genes for this avian IFN have been cloned only recently.[7] They are also present in bony fish,[8,9] reptiles,[10] and, of course, mammals.[11] Their evolution is of considerable interest, not only because the family is large and of relatively ancient origin but also because many of the subtypes themselves are encoded by multiple genes, thereby raising the question of whether the various gene products have acquired different functions. Evolutionists generally agree that

[1]The nomenclature for IFN genes and proteins used herein follows the recommendations of the Nomenclature Committee of the International Society for Interferon and Cytokine Research as described by Diaz et al. (*J. Interferon Cytokine Res.* **16**, 179–180, 1996) and Allen and Diaz (*J. Interferon Cytokine Res.* **16**, 181–184, 1996).

[2]Department of Animal Sciences, University of Missouri, Columbia, MD 65211.
[3]Department of Medicine, Duke University Medical Center, Durham, NC 27710.
[4]Gemini Technologies, Inc., Cleveland, OH 44106.

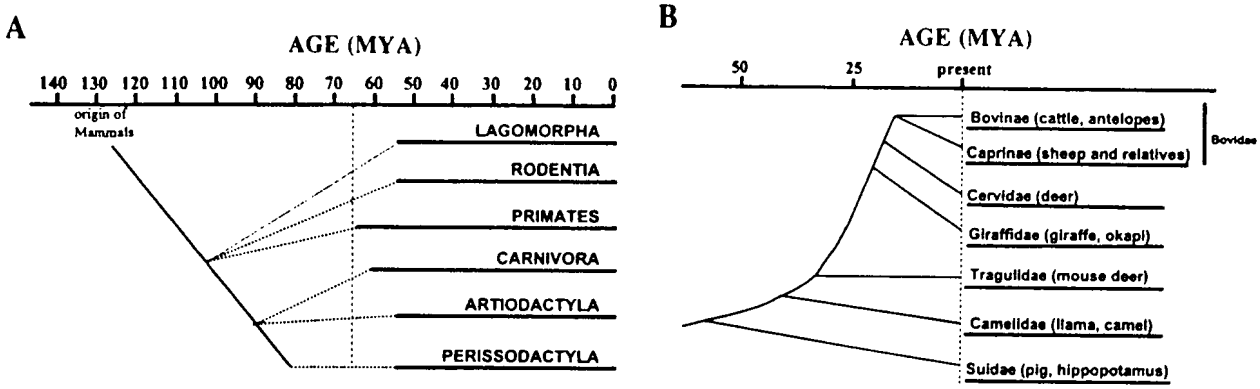

FIG. 1. (A) Phylogenic tree showing possible evolutionary relationships of the main orders of mammals in which IFN have been described. The broken lines indicate where the fossil record is either weak or incomplete. Only for the Perissodactyla is there a fairly complete record. The link between the Lagomorpha and the Rodentia (shown with a dotted line) is controversial. The broken vertical line represents the Tertiary-Cretaceous boundary. (B) Likely phylogenic relationship among the main suborders and families of the Artiodactyla. Note that the Cetacea (whales and their relatives), which are not shown here, are regarded by many authorities to be a suborder within the Artiodactyla and to have diverged from the lineage leading to the suborder Ruminantia about 45 million years ago (MYA).

large multigene families do not persist for long by chance and that superfluous members would be either rapidly eliminated or converted to pseudogenes unless they conferred some selective advantages.[11,12]

In this review, we have attempted to provide an overview of the evolutionary relationships among the type I IFN. Some topics, particularly the evolution of the human *IFNA*, are presented briefly because the likely lineage of these genes has been reviewed recently.[13,14] Instead, we have chosen to explore more fully the origins and relationships of some of the lesser-studied IFN genes, including those encoding the IFN-δ (*IFND*), IFN-ω (*IFNW*), and IFN-τ (*IFNT*). In addition, new data are presented on the evolution of the multiple IFN-β gene (*IFNB*) family of cattle and related species. To assist the reader who is not familiar with the phylogeny of mammals, we have provided a simplified phylogenic tree (Fig. 1A), including all of the mammalian orders in which type I IFN have been described. The Artiodactyla have been subdivided further in Figure 1B to encompass suborders, families, and subfamilies discussed in this review.

ANCESTRY OF THE TYPE I MAMMALIAN IFN

IFNA/IFNB *divergence*

The *IFNA* and *IFNB* genes are found in all mammals where they have been sought. Within a single species, the two gene families display approximately 45% nucleotide sequence identity, a value that translates to about 30% amino acid identity at the protein level (Table 1). Estimates of the time of *IFNA/IFNB* divergence have varied widely, ranging from as recently as 150 million years ago (MYA) to 500 MYA.[14–17] These discrepancies seem to have arisen in part from the kinds of assumptions made about rates of past nucleotide substitution and also from the methods used to make the calculations. Rates of synonymous substitution (i.e., mutations that do not result in amino acid substitution) are less subject to selective elimination and are generally considered to provide a better clock than nonsynonymous changes. However, these rates may vary among different mammalian orders[18] and presumably could have changed with time.[19] In addition, when large evolutionary distances are involved, synonymous sites become saturated, and appropriate corrections for multiple changes at single sites cannot be made. Finally, in the case of the IFN and other multiple gene families, there is invariably some uncertainty about the extent to which gene conversions (i.e., homologous recombination events that can replace an existing gene or part of a gene with a homolog) have blended coding regions over time. Such events may be particularly difficult to detect if they occurred in the distant past. In his 253-MYA estimate of the time of *IFNA/IFNB* divergence, Hughes[14] was compelled to base his calculations on nucleotide substitutions at nonsynonymous sites and assumed that gene conversion events were rare. Although

TABLE 1. PAIRWISE COMPARISONS OF AMINO ACID SEQUENCES AMONG TYPE I IFN FROM CATTLE

n	*IFN subtype*	*GenBank Accession No.*	*1*	*2*	*3*	*4*	*5*	*6*	*7*
			Similarity (%)						
1	IFN-β	M15477		84.4	28.8	29.8	28.3	29.8	27.7
2	IFN-β	M15478			31.5	31.5	33.2	34.8	34.2
3	IFN-α	M11001				92.6	53.2	50.0	49.5
4	IFN-α	M29314					50.0	46.3	45.7
5	IFN-ω	M11002						73.9	72.8
6	IFN-τ	M60913							97.4
7	IFN-τ	M31557							

his value be the best estimate yet, it still remains an approximation and may have to be revised as additional IFN genes are analyzed.

The databank accession numbers for type I IFN are given in Table 2.

The type I avian genes

Figure 2 is a phylogram based on amino acid differences between avian IFNs[7,20,21] and the three most ancient subtypes of mammalian type I IFNs (α, β, and δ), which are represented here by porcine sequences, as IFN-δ has been described only in the pig.[22] Because of the time scales involved, the replacement in the algorithm of the Po-IFN-α and Po-IFN-β sequences with either human, bovine, or mouse sequences does not change the phylogram significantly, nor does it alter any conclusions that can be drawn from it. The data show that the ancestral type I gene duplicated to provide the precursors to the modern day mammalian type I IFN well after birds and mammals diverged, probably about 300 MYA.

To what extent the avian genes have undergone duplications beyond those shown in Figure 2 will not be known until more genes are sequenced, but it is clearly inappropriate to claim that the chicken IFN-α and IFN-β are homologs of mammalian IFN-α and IFN-β. The former originated in a separate series of duplication events that occurred independently from those that gave rise to the mammalian *IFNA* and *IFNB*. As a consequence, a different nomenclature is needed for the avian IFN.

No attempt has been made to place the single known fish IFN sequence[9] in the phylogram shown in Figure 2 because it differs so extensively from the other type I IFN. Figure 2, however, does emphasize the ancient origins of the type I IFN

TABLE 2. DATABANK ACCESSION NUMBERS FOR TYPE I IFN

Interferon	*PIR*	*Swiss protein*	*GenBank*	*Interferon*	*PIR*	*Swiss protein*	*GenBank*
Human				Bovine			
α13	S41196		X75934	ω1	B23285		M11002
α1	A01826		J00210	Ovine			
α5		P01569	X02956	ω	I47070		M73245
α6	A23753		X02958	ω	A61403		
α14	A92916		J00214	Ovine			
α2	A93234	P01563	V00549	τ(p3)			X56341
α8	D23753		K01900	Caprine			
α7	A01831		M34913	τ	I46272		M73243
α17	01835		V00532	Ovine			
α4	E23753		X02955	τ(oTP-1)	I47098		M73242
α10	A01830		V00551	τ(p6)	A61455		X56343
α21	A01832		J00212	Musk-ox			
α16	G23753		M28585	τ			M73244
Equine				Giraffe			
α4	D24912		M14543	τ			U55050
α3	C24912		M14542	Bovine			
α1	A24912		M14540	τ	A40068		M31556
α2	B24912		M14541	Human			
Porcine				ω1	A93070		M11003
α1		P49879	M28623	Rabbit			
Bovine				ω	I46972		S68997
αD	D26028		M10955	Equine			
α1	A23285		M11001	ω1	F24912		M14545
αB	B26028		M10953	ω1	E24912		M14544
αC	C26028		M10954	Porcine			
αA	A26028		M10952	δ	A48772		Z22706
Feline				β			M86762
α	JS0664		S62636	Equine			
Canine				β	G24912		M14546
α2	I46206		M28626	Human			
α1	I46204		M28624	β1	A93721		M28622
Murine				Bovine			
α6	D23087		X01972	β3	A01841		M15479
α5	C23087		X01971	β2	A01840		M15478
α1	A01836		X01974	β1	A01842		M15477
α4	B23087		X01973	Murine			
α2	A01837		X01969	β	S02020		X14029
Porcine							
ω3	S23712		X57194				
ω5	S23711		X57196				
ω4	S23710		X57195				

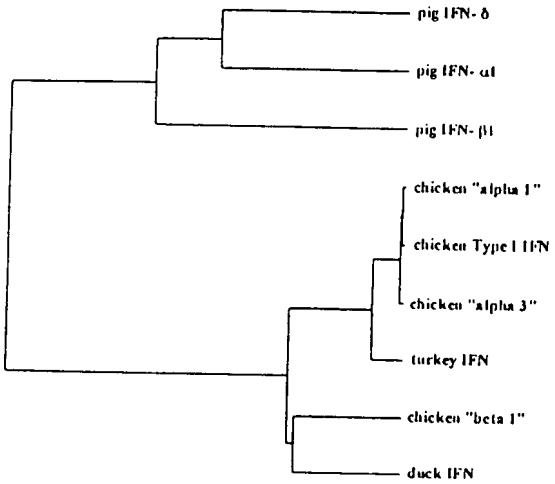

FIG. 2. A Growtree phylogenetic tree based on amino acid sequence identities showing relationships between avian and mammalian type I IFN. The tree was constructed by performing pairwise alignments by means of the Wisconsin GCG programs Distances and Growtree. The lengths of the branches are proportional to the degree of amino acid diversity. Protein databank symbols are listed in Table 1. GenBank Accession numbbers: Z22706, pig IFN-δ; M28623, pig IFN-α1; M86762, pig IFN-β1; X92476, chicken "alpha 1"; U07868, chicken type I IFN; X92478, chicken "alpha 3"; U28140, turkey IFN; X92479, chicken "beta 1"; X84764, duck IFN.

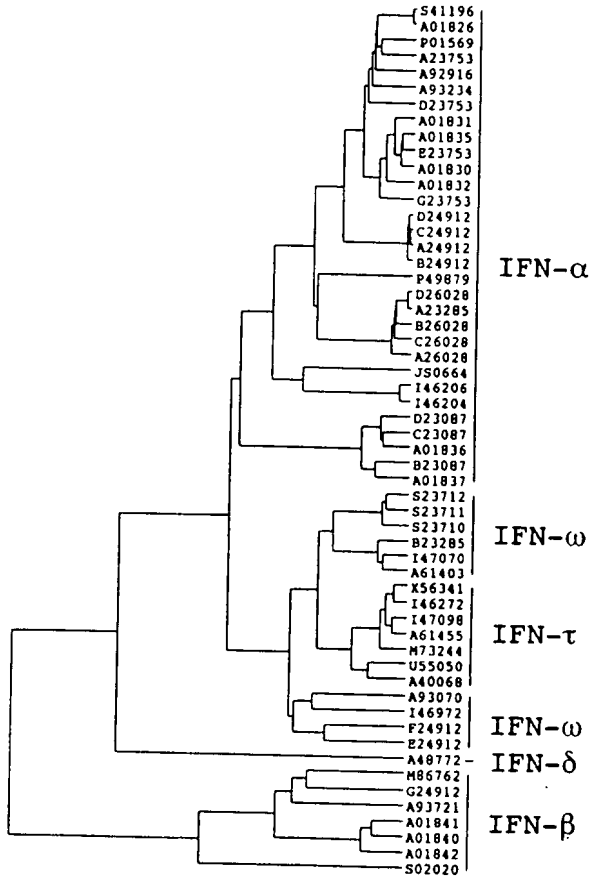

FIG. 3. A Growtree phylogenic tree based on amino acid sequence identities for type I IFN from mammalian species from which type I IFN genes or cDNA have been cloned and sequenced. Although all the known human IFN-α are shown, there has been no attempt to include all known IFN sequences from all species in the comparison. The IFN are listed by their protein databank accession numbers (PIR or Swiss Protein). Table 2 lists databank accession numbers for the IFN in Figure 3.

and is consistent with their presence in reptiles and even amphibians.

The evolution of the IFND

Porcine IFN-δ was identified in the secretions of the pig trophoblast (preplacenta) during early pregnancy.[22] It is shorter than other type I IFN, the mature protein being only 149 amino acids in length, but would appear to retain the signature fold of IFN-α and IFN-β, bind to the type I receptor,[23] and induce an antiviral response in target cells.[24] IFN-δ does not seem to be encoded by a virally inducible gene,[22] although its promoter sequence retains some similarities to those of the *IFNA* and *IFNB*. Unlike the IFN-τ discussed later, which have a well-defined physiologic role in the pregnancy of large ruminant species, the function of Po-IFN-δ is unclear. Curiously, it is expressed by trophoblast at an identical time in pregnancy as type II IFN (IFN-γ).[22,25]

Calculations based on coding sequence divergence suggest that *IFND* and *IFNA* diverged 180 MYA[22] and well before the appearance of mammals. The presence of these genes in the pig, a monogastric species whose ancestor diverged from other Artiodactyla approximately 55 MYA, suggests that the *IFND* could potentially be represented in all the major mammalian orders. However, little new information has appeared on these interesting molecules.

DIVERSITY AND DUPLICATION WITHIN THE *IFNB*

IFNB genes have been cloned from several mammalian species (Fig. 3). Whereas Wilson et al.[26] detected only a single gene by Southern blot hybridization of restriction enzyme-digested DNA from primates and rodents, a double band, suggestive of an *IFNB* duplication, was noted with rabbit (Lagomorpha) and lion (Carnivora) DNA. Even within the same order, the number of *IFNB* genes can vary greatly. The pig (family Suidae) has only a single *IFNB*,[27] whereas cattle, also artiodactyls but of more recent lineage, have several[26] including three that have been cloned and sequenced from the cow (*Bos taurus*).[28]

Figure 4 illustrates a typical difficulty in interpreting Southern genomic blots where there are multiple, probably recently duplicated genes. The majority of the Bovidae species shown appear to have at least six *IFNB* in restriction fragments that hybridize to an *IFNB* probe, whereas the nyala (family Bovidae) and giraffe (Giraffidae) each show several faint and one intense band when EcoRI-digested DNA is analyzed. As these lanes were loaded with approximately equivalent amounts of DNA and the probe used (a labeled IFN-β cDNA) did not hybridize to bovine *IFNA*, *IFNW*, and *IFNT* sequences (data not shown), it seems more likely that the intense band represents

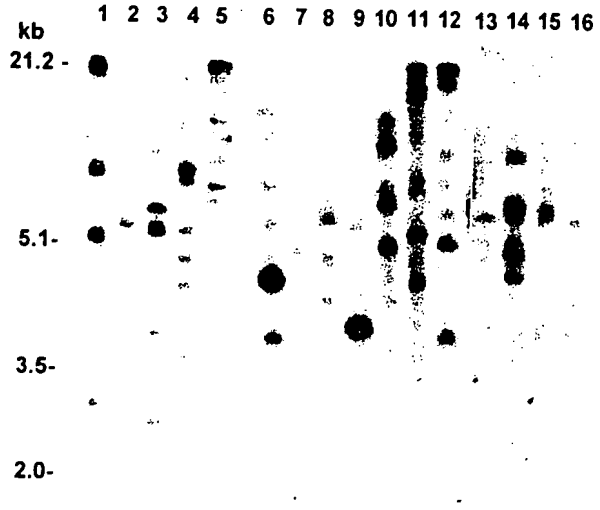

FIG. 4. Southern genomic blots of EcoRI-cut genomic DNA from Artiodactyla species. Blots wre hybridized with a probe for the gene for bovine IFN-β3. DNA size markers in kilobases are on the left. The lanes are from the following species: lane 1, moose; lane 2, mule deer; lane 3, white tail deer; lane 4, impala; lane 5, springbok; lane 6, giraffe; lane 7, mule deer; lane 8, duiker; lane 9, nyala; lane 10, gnu; lane 11, yak; lane 12, cape buffalo; lane 13, takin; lane 14, dall sheep; lane 15, Suffolk sheep; lane 16, hippopotamus.

FIG. 5. Southern genomic blot of *Bos taurus* genomic DNA cut with various restriction enzymes and probed with a probe for the gene for bovine IFN-β3. Lane 1, BamHI; lane 2, Hind III; lane 3, EcoR1; lane 4, EcoRV; lane 5, Bg1II; lane 6, BstXI.

several structurally similar genes that were not discriminated by EcoRI digestion. In Figure 5, calf thymus DNA was digested with several restriction enzymes that did not cleave within the probe sequence and was then probed for *IFNB*. One interpretation of lanes 1, 5, and 6 (Fig. 5) might again be that there is a single *IFNB* represented by the strong band, plus a few distantly related genes that hybridize weakly to the probe. On the other hand, the BglII lane contains what appears to be three poorly resolved doublets along with a more intense 3.7-kb band. The latter has been subcloned, and the *IFNB* gene in individual colonies has been sequenced. To date, at least three distinct *IFNB*, similar but not identical in sequence to the *IFNB3* gene characterized by Leung et al.,[28] have been found (data not shown). There may, therefore, be 10 or more *IFNB* in *Bos taurus*, including several genes that have duplicated quite recently.

Together these studies indicate that although the *IFNB* gene has remained as a single entity over considerable lengths of evolutionary time in some mammalian lineages, there have been sudden and quite recent bursts of duplication in others. It would appear that once a duplication has occurred, further amplification becomes much easier, most likely because the possibility of homologous recombination and unequal crossing-over events is enhanced.

DIVERSITY AND DUPLICATIONS WITHIN THE *IFNA*

Multiple *IFNA*s have been detected in every mammalian species where they have been sought, and individual genes have been cloned and sequenced from human, mouse, cat, dog, rabbit, horse, pig, and cow, species that represent a wide range of mammalian orders (Fig. 3). Only in the human is it likely that all of the *IFNA* genes that make up the cluster have been identified.[14] In the early 1980s, the realization that there were numerous *IFNA* in species as distinct as the human and mouse prompted the suggestion that a family of multiple genes must have existed before the radiation of the major mammalian orders.[29] Subsequent comparisons of gene and amino acid sequences have indicated that there is significantly more variation between species than within a single species.[14] One interpretation of such data is that the majority, if not all, of the duplication events occurred after the separation of the main mammalian orders.

As discussed earlier, however, unequal crossing-over events, involving the coding regions, could possibly blend genes, thereby masking their evolutionary history and enhancing interspecies differences. Hughes,[14] in his detailed phylogenic analysis of human genes, and Diaz et al.,[13] in their mapping of the entire *IFNA/IFNW/IFNB* locus of the human, both agree that such recombination events have probably occurred but have not contributed greatly to the evolution of the majority of the *IFNA* genes as they exist today. Overall, the evidence appears to favor the view that diversification of the *IFNA* gained momentum after the initial radiation of the main mammalian orders. Such a conclusion has three obvious implications. First, the type I locus will not be arranged identically in different taxonomic groups of mammals (i.e., precise synteny will not be observed). Second, the numbers of *IFNA* will differ among such groups. Third, duplicated genes will likely have evolved distinct functions. Their products might well exhibit different biologic activities, and the genes themselves might differ, possibly subtly, in the manner in which they are transcriptionally activated in response to external cues. In the human, it is clear that several of the *IFNA*

duplications occurred quite recently[13,14] and the majority within the last 30 million years.[30]

THE EVOLUTION OF THE *IFNW*

The *IFNW* and their corresponding gene products, the IFN-ω, were first described in 1985 after human and bovine genomic and human leukocyte cDNA libraries were screened under relatively nonstringent conditions with *IFNA* probes. These IFN, originally named IFN-αII by one group[31] and IFN-ω by another,[32] are now recognized as sufficiently distinct from the IFN-α to deserve the separate ω-subtype status.[3] There are multiple *IFNW* gene loci in human,[13,31] but only one is known to be functional. In the cow there are also many genes,[33] possibly 20 or more.[34] The IFN-ω are characterized by being six amino acids longer at their carboxyl-terminal than the IFN-α and, because of anomalous signal peptide cleavage, can also possess an extra two residues at their amino-termini.[35] Despite these structural differences, Hu-IFN-ω1 appears to possess comparable biologic activity to many Hu-IFN-α[35] and to be virally inducible in leukocytes.[31] Its particular function is not understood.

Since their initial discovery in cattle and humans, *IFNW* have been cloned from sheep, pig, horse, and rabbit (Fig. 3), and their presence has been detected by Southern blotting analysis in many other species as well (Fig. 6). Capon et al.[31] estimated that *IFNA* and *IFNW* diverged between 116 and 132 MYA. Using a different means of calculation, Hughes derived a time of 129 MYA.[14] Both these estimates suggest that the two subtypes originated before the main radiation of mammals. It is curious, therefore, that *IFNW* have been reported to be absent in the dog[36] and have not been described in the mouse, a relatively well studied species. In the case of the dog, a gene remnant, part of a putative promoter region, was cloned, suggesting that the genes might have once existed and subsequently became lost. The presence of six pseudogenes and only a single functional *IFNW* in the human[13] also implies that these genes, once duplicated, had neutral or even negative value to the species.

There is no estimate of how many of the apparently numerous *IFNW* found in species other than human are functional. Of those listed in Figure 3, all have uninterrupted open reading frames (CRF) encoding a full-length protein, but all except the human were cloned from genomic DNA and not from RNA transcripts. They are an unusually diverse group of genes, and in view of the complexity of Southern blots (Fig. 6B), many more remain to be described. It would not be surprising if the *IFNW* have to be subdivided into additional subtypes as additional members are cloned. Subtypes are usually defined rather arbitrarily by structural distinctiveness, based on amino acid divergence and antigenicity, biologic function, and site of expression.

Hughes,[14] in his analysis of the *IFNW*, concluded that these genes duplicated relatively early in their existence and that the modern day forms reflect these early duplication events. He based his conclusion on the fact that nucleotide differences among the *IFNW* within a species were as great or greater than between species. He failed, however, to take into consideration the fact that the *IFNW* and *IFNT* are distinct subtypes and should be analyzed separately. In addition, his analysis included an ovine gene[37,38] that corresponds very closely in sequence to rabbit *IFNW*s,[39] so closely, in fact, that it has been suggested that the gene originated in the rabbit and reached the sheep through some form of horizontal transfer, most likely involving a retrovirus.[38] A follow-up analysis of *IFNW* sequences, ignoring the anomalous ovine/rabbit *IFNW*, has shown that the similarities of *IFNW* from the same species, for example, in sheep, rabbit, and pig, are high and that there are larger differences between species than within them.[40] Even though some of the duplication events may be quite ancient, such as the one that separated the two known horse *IFNW* (Fig. 3),[41] it would appear that most of the *IFNW* duplication occurred after the establishment of the main orders of mammals and that many are of quite recent origin.

THE EVOLUTION OF THE IFN-τ GENES (*IFNT*)

The IFN-τ, like the IFN-ω from which they evolved,[33,40] possess a six amino acid extension at their carboxyl end when they are aligned with the IFN-α. They were identified in the late 1980s as the factors produced by the developing trophoblast (preplacenta) of cattle and sheep that were responsible for extending the life span of the corpus luteum during early pregnancy, thereby preventing continued ovarian cyclicity.[42] In this regard, they appear to be essential for pregnancy maintenance and to have assumed a very different role than their closest relatives, the IFN-ω and IFN-α.

The reproductive function of the IFN-τ has been widely misunderstood within the field of IFN biology. It has been argued, for example, that IFN cannot play an essential role in pregnancy because mice lacking components of the type I receptor[43] or having an impaired IFN signal transduction pathway[44] have normal fertility. What has not been appreciated is that the mechanisms whereby the embryo communicates with the mother and maintains corpus luteum function in mice are very different from those used in hoofed species, such as the cow or sheep.[45] Humans and related primates use yet different strategies, but, as in mice, there is no apparent involvement of IFN of any kind. Indeed, as discussed in further detail later, the *IFNT* probably do not exist outside the suborder Ruminantia.

Gene number

Genes for the IFN-τ have been identified by Southern genomic blotting in cattle, sheep, musk oxen, goats, and gazelle (all of which are in the Bovidae), giraffe (Giraffidae),[33] and a variety of deer (Cervidae) (unpublished observations) (Fig. 6). With the exception of the giraffe, where it is unclear if there is a single gene or a group of very closely related genes (Fig. 6), all the ruminants mentioned appear to possess several *IFNT*. Estimates of gene number for cattle have ranged from a minimum of 4[33] to at least 10.[34] In sheep, several distinct genes have been cloned (listed in ref. 46), as well as at least one pseudogene,[33] but an exact count will probably require that the entire locus be mapped. Although all *IFNT* sequences are well conserved, most likely because the genes evolved so recently, the degree of nucleotide sequence identity of genes within a species is greater than between species.[40] Again, it

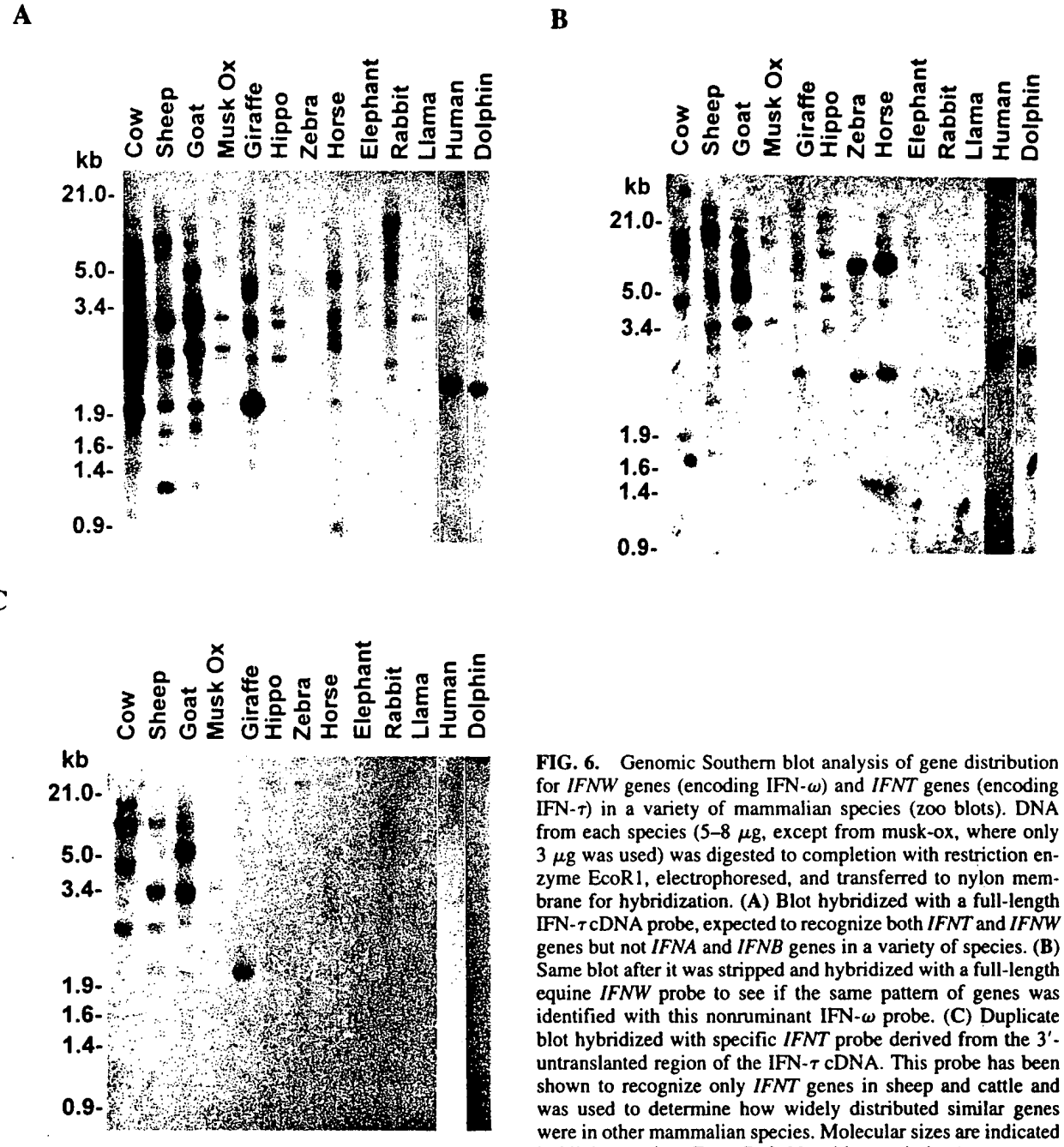

FIG. 6. Genomic Southern blot analysis of gene distribution for *IFNW* genes (encoding IFN-ω) and *IFNT* genes (encoding IFN-τ) in a variety of mammalian species (zoo blots). DNA from each species (5–8 μg, except from musk-ox, where only 3 μg was used) was digested to completion with restriction enzyme EcoR1, electrophoresed, and transferred to nylon membrane for hybridization. (A) Blot hybridized with a full-length IFN-τ cDNA probe, expected to recognize both *IFNT* and *IFNW* genes but not *IFNA* and *IFNB* genes in a variety of species. (B) Same blot after it was stripped and hybridized with a full-length equine *IFNW* probe to see if the same pattern of genes was identified with this nonruminant IFN-ω probe. (C) Duplicate blot hybridized with specific *IFNT* probe derived from the 3'-untranslanted region of the IFN-τ cDNA. This probe has been shown to recognize only *IFNT* genes in sheep and cattle and was used to determine how widely distributed similar genes were in other mammalian species. Molecular sizes are indicated in kilobase pairs. (From Ref. 33, with permission.)

would appear that the *IFNT* have continued to duplicate independently in different lineages of the Ruminantia.

Time of origin

When two genes have diverged from a common ancestor relatively recently, the differences between the two sequences provide a more accurate representation of evolutionary distance than if the event occurred many millions of years ago[47]—hence, the uncertainty of the time of *IFNA/IFNB* divergence. Table 3 is a matrix of corrected distances (i.e., number of substitutions per 100 bases) of representative *IFNW* and *IFNT* genes within the suborder Ruminantia. Some selection of gene sequences, particularly for the *IFNT*, was made in these calculations because there are over a dozen sequences for Ov-IFN-τ alone in GenBank,[46] and it remains unclear how many of these are allelic. The ones chosen were sufficiently different that they likely represent distinct genes. Extending the number of sequences analyzed does not alter the conclusions that can be drawn from Table 3.

The distances between the *IFNT* genes of cattle (subfamily Bovinae) and those of sheep goat and musk-ox (subfamily Caprinae) average 10.9 per 100 bases as calculated by the Kimura two-parameter method.[47] It is generally accepted from the fossil record that the ancestors of the present day Bovinae and Caprinae diverged about 20 MYA.[48] If it is assumed that

TABLE 3. DISTANCES BETWEEN NUCLEIC ACID SEQUENCES IN CODING REGIONS OF *IFNT* AND *IFNW* IN RUMINANTIA[a]

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
1 Ov*IFNW1*	0.00	5.87	6.06	7.37	17.14	18.23	16.68	17.34	16.56	15.71	16.96	16.33
2 Ov*IFNW2*		0.00	6.61	6.43	16.92	18.00	16.91	17.34	15.92	15.49	16.32	18.35
3 Ov*IFNW3*			0.00	8.51	17.33	17.97	16.43	17.06	16.96	16.11	17.14	18.26
4 Bo*IFNW1*				0.00	17.35	18.22	16.47	17.80	16.57	16.14	17.43	18.36
5 Ov*IFNT1*					0.00	1.91	3.68	6.62	11.68	10.69	11.87	14.18
6 Ca*IFNT1*						0.00	3.50	6.05	12.28	11.27	12.46	15.23
7 Ov*IFNT2*							0.00	4.59	10.88	9.89	11.06	13.34
8 Mo*IFNT*								0.00	12.11	11.11	12.29	15.30
9 Bo*IFNT1*									0.00	1.03	1.91	11.61
10 Bo*IFNT2*										0.00	1.55	10.82
11 Bo*IFNT3*											0.00	11.99
12 Gi*IFNT*												0.00

[a]Distances are number of substitutions per 100 bases calculated by the Kimura 2-parameter method[47] by using the Genetic Computer Group (University of Wisconsin) sequence analysis software package (Version 7.1). The access numbers for IFN used in this comparison are X59067 (Ov*IFNW2*), M73245 (Ov*IFNW3*), M11002 (Bo*IFNW1*), X56345 (Ov*IFNT1*), M73243 (Ca, caprine *IFNT1*), X56346 (Ov*IFNT2*), M783244 (Mo, musk-ox *IFNT*), M31557 (Bo*IFNT3*), M31558 (Bo*IFNT1*), M60913 (Bo*IFNT2*), and U55050 (Gi, giraffe *IFNT*). Ov*IFNW1* is from Charlier et al.[39] (Data from Ref. 40, with permission of Academic Press.)

the *IFNT* genes evolved at the same rate in both lineages, the base substitution rate has been 0.271 ± 0.004/100 bases/MY. A quite similar rate (0.275 ± 0.008) can be calculated for the giraffe, whose ancestors diverged from Bovidae approximately 24 MYA.

If similar calculation are performed on the *IFNW*, the closest known relatives of the *IFNT*, the results are different. The substitution rate within Bovidae is 0.186 ± 0.015 over 20 MY and is 0.179 ± 0.003 if the bovine and ovine *IFNW* are compared with those of pigs, whose ancestors diverged from the precursors of the modern ruminants approximately 55 MYA. Evidently, the *IFNT* genes are evolving almost 50% faster than the *IFNW*. If the base substitution rates noted in Table 3 are assumed to have been maintained in the two sets of genes after they diverged from the common *IFNT/IFNW* ancestor, the branch point occurred 36.5 ± 0.24 MYA.[40] Therefore, the first *IFNT* evolved from its progenitor *IFNW* gene soon after the appearance of the suborder Ruminantia.[48] The prediction is entirely consistent with experimental data that *IFNT* are not present in pigs (suborder Suiformes, family Suidae) or llamas (suborder Tylopoda, family Camelidae).[33] It is possible, but unlikely, that they will be found in the whales and related species (Cetaceae), which are not considered to be very close relatives of Ruminantia. The Tragulidae (mouse deer) are better candidates (Fig. 1B), having originated around the time of *IFNT* divergence from the *IFNW*.

Structural evolution of the 5′-untranslated regions (5′-UTRs)

The first 130 bases of the 5′-UTRs of the *IFNT* are highly conserved among sheep, goats, musk-oxen, cattle, and giraffes.[33,49] Where a more extensive upstream region of cattle and sheep genes has been sequenced, conservation is evident up to about 400 bases beyond the transcription start site and is then lost. The evolutionary distances between such bovine and ovine genes within this putative promoter region are comparable to those calculated for the coding region (Table 4) (i.e., they are equally conserved and have evolved at similar rates). The *IFNW* promoters are also relatively well conserved, not only between cattle and sheep but also between ruminant species and pigs (data not shown). In the case of the *IFNW*, however, the rate of substitution has been higher in the 5′-UTR than in the coding region (Table 4).

The promoter sequences of the *IFNT* and *IFNW* bear only a

TABLE 4. DISTANCES IN NUCLEOTIDE SEQUENCES

	Average Distances[a] (± SEM)		
	IFNW/IFNT	*IFNT/IFNT*	*IFNW/IFNW*
Promoter	50.78 ± 0.78	9.02 ± 0.85	11.56 ± 0.91
Coding region	16.69 ± 0.11	10.85 ± 0.16	7.44 ± 0.60
3′-UTR	43.97 ± 0.75	10.05 ± 0.20	8.58[b] and 31.0[c]

[a]Distances are number of substitutions per 100 bases calculated by the Kimura 2-parameter method.[47] Cattle sequences are compared with sheep, musk-ox, and goat sequences to obtain the distances of *IFNT(IFNT/IFNT)* and *IFNW (IFNW/IFNW)* between Bovinae and Caprinae. Both intraspecies and interspecies comparisons are included in calculating the average distance between *IFNT* and *IFNW* within the family Bovidae. The promoter region used in the comparison is about 130 bp upstream of the transcription start site, and the 3′UTR is about 300 bp downstream of the stop codon. The distance[b] between the bovine *IFNW* and Ov*IFNW2* was very different from that[c] between Bo*IFNW* and Ov*IFNW1* when 3′UTR comparisons were made. The access numbers for IFN used in this comparison are M11002, M31557, M31558, M60908, M60913, M73241, M73242, M73243, M73244, M73245, M88771, M88772, X56342, X56345, X56346, X59067, and X65539. The sequences for *IFNT* and *IFNW* from Charlier et al.[40] are also included in the comparison; they are not in GenBank. (Data from Ref. 40, with permission of Academic Press.)

limited sequence resemblance to each other,[33,40] and the calculated distance, 50.78 (Table 4), corresponding to a base substitution rate of 0.71/100 bases/MY, is improbably high. The actual substitution rate within *IFNT* promoters over the 24 MY since the beginning of radiation within the true (pecoran) ruminants (Ruminantia) has been much more modest (0.23/100 bases/MY). Such a substitution rate could not account for the difference observed between the *IFNT* and *IFNW* genes. Presumably, a series of more abrupt genetic changes, such as deletions and recombination events within the 5′-UTR, accompanied the first emergence of the *IFNT* gene as a distinct subtype. The acquisition of a unique promoter may have been the triggering event that provided placental expression and abolished viral inducibility.

Structural evolution of the 3′-UTR

The entire 3′-UTR of about 300 bp of the *IFNT* is as conserved as the 5′-UTR and ORF (Table 4). The average evolutionary distance (number of substitutions/100 bases) between bovine and ovine *IFNT* in this region is only 10.05 ± 0.20. The 3′-UTRs of the *IFNT* and *IFNW* genes share about 80% sequence identity over the first ~120 bases beyond the stop codon but then diverge markedly (<65% further downstream). Even this degree of identity is probably due to the fact that both sets of genes are AT rich in this region.[50] It is for this reason that probes prepared from the 3′-UTR can be used to distinguish *IFNT* and *IFNW* genes by Southern genomic blotting, whereas probes from the ORF cannot (Fig. 6).[33] These observations strongly suggest that the duplication event that initially gave rise to the *IFNW/IFNT* split was caused by acquisition not only of a specialized promoter but also of a unique region ~130 bp downstream of the stop codons.

Acquisition of the signature six amino acid carboxyl tail of IFN-τ and IFN-ω

It is unclear how the progenitor gene of the present day *IFNT* and *IFNW* gained six extra codons when it was created, presumably by a duplication, from an IFNA gene. In Figure 7, the base sequences for the genes of Bo*IFNT1*, Bo*IFNW1*, and Bo*IFNA1* in the region close to the ends of the ORFs are compared. It is clear that a considerable degree of nucleotide sequence identity has been maintained for Bo*IFNW1* and Bo*IFNT1* both up to and after the stop codon. In contrast, the similarity of these two genes with Bo*IFNA1* breaks down even before the *IFNA1* stop codon. Because there are no obvious base insertions or deletions that can account for the extension in the ORF for *IFNW* and *IFNT*, it may be that the mutational event can no longer be traced of the length of time that has elapsed since its occurrence. An alternative explanation is that a recombination event occurred near the end of the ORF of the precursor *IFNA* to provide a novel 3′-end to the new gene. One possible location for the event to have occurred would be at or near base position 555 (codon 185), which marks the site where major genetic divergence begins.

```
boIFNT1 CAGAATGGAG ATGATGAGAG CCCTCTCTTC ATCAACCACC TTGCAAAAAA 550
boIFNW1 ····C····A ··C·····T ··T·G····· ·····G· ·····G··· 550
boIFNA1 ····GCAC·A G·C····· ··T····· C·····A·A· ·····GG·G· 550

boIFNT1 GGTT-A--AG AAA-GATGGG TGGAGATCTG AACTCACTTT GAGATGACTC 596
boIFNW1 ·····-···· ··T·····A ·····C··· ··A····C·· ··C····· 596
boIFNA1 ·A··C·GG·· ···G··CTGA CAC·C·C··· GTTCA··ACG ··A····T·· 600

boIFNT1 TCGCTGACTA AGATGCCACA TCACCTTCGT ACACTCACCT GTGTTCATTT 646
boIFNW1 ··A····· ·····C·· ···T···T·C ·····T·· ···GC····· 646
boIFNA1 ··A·G·C··· ·C·GA····· CTT···C·TG CGCTG·CATG TG·AAG·C·C 650
```

FIG. 7. Alignment of nucleotides surrounding the stop codon for bovine *IFNT1*, *IFNW1*, and *IFNA1* genes. The Bo*IFNT1* sequence was derived from Imakawa et al.,[50] (GenBank Accession M31558), and Bo*IFNW1* and Bo*IFNA1* sequences were derived from Capon et al.[31] (GenBank Accession M11002 and M11001, respectively). Underlined sequences represent stop codons (TGA). Sequences were numbered according to number of bases from the translational start site. The Bo*IFNT* served as the consensus sequence for comparisons.

THE CHROMOSOMAL LOCATION AND LINKAGE OF HUMAN TYPE I GENES

All of the known human type I IFN genes are clustered on the short arm of chromosome 9 within 400 kb of each other (Fig. 8).[13] The single *IFNB* gene is placed at the distal end relative to the centromere. There are seven *IFNW* genes, only one of which is functional, 13 functional *IFNA*, a single *IFNA* pseudogene (*IFNAP22*), and three additional pseudogenes (*IFNP23*, *IFNP12*, *IFNP11*) with only a limited resemblance to the *IFNA*.

The arrangement and sequences of these genes allowed Diaz et al.[13] to infer how the locus might have evolved. For example, the genes on the proximal side of *IFNP12* are transcribed toward the centromere, whereas those on the distal side have an opposite orientation. Therefore, it seems likely that early in the evolution of the locus, a cluster of a few genes was duplicated and inverted. As a result, *IFNA2* and *IFNA8* are likely to be homologs. The close sequence similarities among the *IFNA*

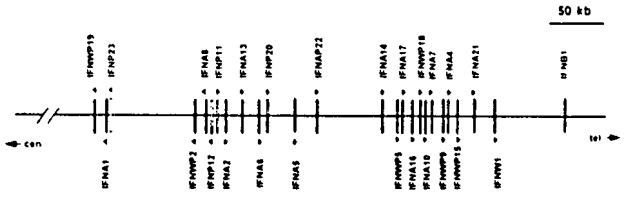

FIG. 8. Map of the IFN gene cluster showing the locations of the different *IFN* genes and pseudogenes as vertical bars. The arrowheads show the directions of transcription inferred for each gene. The gene for *IFNW1*, the only *IFNW* that is transcribed, is distally placed relative to the centromere (cen) and located between the genes for IFN-α21 (*IFNA21*) and IFN-β (*IFNB*), which is closest to the telomere (tel). Pseudogenes are designated with a P, for example, *IFNWP15* and *IFNAP22*. The IFN-related pseudogenes are designated by open bars. (From Diaz et al.,[12] with permission.)

genes in the distal group suggest that they arose from a more recent series of duplications than the one that originally gave rise to the proximal group.[13]

The arrangement gives little clue as to how the functional *IFNW1* gene, which is placed distally in the cluster, arose. Of the six pseudogenes, two (*IFNWP2* and *IFNWP19*) are placed in the proximal cluster nearest the centromere, the remaining four in the distal cluster of oppositely transcribed *IFNA* genes. Some of the duplications appear to have involved a repeated cassette of two *IFNA* and one *IFNW* pseudogene. Diaz et al.[13] speculate that the distally placed genes *IFNPW15* and *IFNA21* are ancestral to these *IFNA/IFNA/IFNPW* units and that a duplication followed by a loss of one *IFNPW* gave rise to the first triplet. They argue that the majority of duplications were mediated by unequal crossing-over events occurring in conserved regions, such as long repetitive elements, between genes and not within the genes themselves.

No other IFN locus has been physically mapped to determine if it is organized similarly to that of the human, although our prediction, based on the hypothesis that most IFN gene duplications are of recent origin, is that similarities will be limited. In cattle, the type I genes appeared to be closely linked on bovine chromosome 8, band 25. A tentative order *IFNA/IFNW-IFNT-IFNB* has been inferred by restriction endonuclease mapping.[34]

ARE THERE HUMAN GENES FOR THE IFN-τ?

In 1994, Whaley et al.[51] reported the cloning of a cDNA from a human placental cDNA library that encoded an IFN with close similarity to ovine and bovine IFN-τ. *In situ* hybridization appeared to confirm that the gene was expressed primarily in cytotrophoblast, although nonspecific hybridization to related transcripts, such as transcipts for IFN-ω and IFN-α, was not ruled out. The discovery of a human IFN-τ was surprising because it implied that the genes arose much earlier in mammalian evolution than could be anticipated from gene and protein sequences.[40] The report was also of particular interest because the prospects for using IFN-τ in treatment of human disease continue to be raised as a result of the apparent low cytotoxicity of these IFNs.[52] A recombinant Ov-IFN-τ has been reported to be as effective as murine IFN-β in reducing the severity of an experimentally induced autoimmune condition in mouse[53] and in treatment of multiple melanoma.[52] There are additional claims that IFN-τ might be especially valuable in inhibiting retrovirus replication, including that of the AIDS virus.[54] Clearly, a human IFN-τ, lacking the potential antigenicity of an ovine protein, would be of value. Despite the initial cloning of a cDNA[51] and the report that there may be as many as seven *IFNT* genes (cited as unpublished results in reference 53) there are reasons to suspect that such human genes do not exist.

An analysis of the inferred amino acid sequence of the human placental IFN-τ shows that it most resembles a bovine or ovine IFN-ω (87%–89%) rather than an ovine IFN-τ (72%–75%). Moreover, no such gene has been identified in the 400-kb type I locus (Fig. 8),[13] although the region does contain seven *IFNW*-like sequences, a numerical value that corresponds to the *IFNT* gene number reported by Soos et al.[53] Attempt in this laboratory to isolate the *IFNT* gene either by screening a human genomic library with *IFNT* probes or by PCR amplification based on the sequence reported by Whaley et al.[51] have either failed or produced gene sequences corresponding to human *IFNW*. Perhaps most importantly, the data presented in this review strongly suggest that the *IFNT* arose quite recently (36 MYA) within the suborder Ruminantia. As a consequence, they could not be expected to exist in primates.

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Address reprint requests to:
Dr. R. Michael Roberts
Department of Animal Sciences
Animal Sciences Center
University of Missouri
920 East Campus Drive
Columbia, MO 65211-0001

Fax: 573-882-6827
E-mail: robertsrm@missouri.edu

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